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Published in:
Photochemistry and Photobiology

DOI:
[10.1111/j.1751-1097.1984.tb05355.x](https://doi.org/10.1111/j.1751-1097.1984.tb05355.x)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1984

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
STAVENGA, DG., FRANCESCHINI, N., & KIRSCHFELD, K. (1984). FLUORESCENCE OF HOUSEFLY VISUAL PIGMENT. *Photochemistry and Photobiology*, 40(5), 653-659. <https://doi.org/10.1111/j.1751-1097.1984.tb05355.x>

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FLUORESCENCE OF HOUSEFLY VISUAL PIGMENT

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(Received 20 March 1984; accepted 14 June 1984)

Abstract—The fluorescence of housefly photoreceptors was studied *in vivo* by using the deep pseudopupil technique. Whereas the rhodopsin R490 of the peripheral reticular cells fluoresces negligibly the metarhodopsin M580 fluoresces distinctly in the red. The newly discovered metarhodopsin M' is produced by intense blue light and can be reconverted into rhodopsin by intense long wavelength light. M' also fluoresces in the red; its excitation spectrum and emission spectrum peak at $\lambda_{\text{max}} \approx 570$ and 660 nm respectively.

Intense ultraviolet light irreversibly reduces the visual pigment fluorescence as well as the broad band autofluorescence ($\lambda_{\text{max}} \approx 470$ nm) originating from non-visual pigments in the fly's eye.

INTRODUCTION

The distinct fluorescence displayed by the rhabdomeres, i.e. the visual pigment containing organelles, of most fly photoreceptor cells can be observed in completely intact, living animals (Franceschini, 1977, 1978, 1983; Stark *et al.*, 1977, 1979; Franceschini *et al.*, 1981a). These observations have provided a new and powerful tool, not only for identifying and characterizing cell types (Franceschini *et al.*, 1981b), but also for studying the photochemistry of the visual process, the theme of the present paper.

It is a well established fact that the main visual pigment of flies, residing in the peripheral rhabdomeres, is a blue-green-absorbing rhodopsin R which is photo-interconvertible with a metarhodopsin M absorbing mainly in the orange wavelength range (Hamdorf, 1979). The λ_{max} (the wavelength of maximal absorption) for the rhodopsin and metarhodopsin of the housefly *Musca domestica* are 490 nm and 570 nm respectively (Minke and Kirschfeld, 1979; Kirschfeld, 1981). Here we demonstrate that housefly metarhodopsin fluoresces and, furthermore, we report the emission and excitation spectra of another visual pigment state coined M' (Franceschini *et al.*, 1981a) which is created at extremely bright light intensities. For preliminary accounts see Franceschini and Stavenga (1981) and Stavenga and Franceschini (1981).

MATERIALS AND METHODS

The measurements were performed with a micro-spectrophotometer equipped with an epifluorescence illuminator (Leitz Orthoplan—Ploem Opak with an MPV-2

or Compact photometer head). The excitation beam was delivered by a 75 W Xe arc or a 100 W Hg arc (Osram) filtered by narrow-band interference filters (Schott, half width ≈ 15 nm) and quartz-neutral density filters. Emission was measured by either an EMI 9558 QB photomultiplier tube (S 20 photocathode) or a Hamamatsu R 928 (broad-band multialkali photocathode) in front of which either a far-red long-pass filter (Wratten 70) or a motorized interference-filter wedge (Schott Veril S-60) was placed.

Preparation of flies. A live intact housefly *Musca domestica*, mutant *white*, was mounted with its head adjusted at the centre of curvature of a goniometer (Leitz-Universal stage), so that a suitable eye region could easily be selected. In order to monitor the metarhodopsin concentration, transmission measurements were performed by delivering yellow "antidromic" light (572 nm) through a thin flexible light-guide pinned into the back of the head capsule. Photopigment conversions were then induced by "orthodromic" (i.e. normally incident) blue or red light, none of which could reach the photomultiplier due to a 572 nm narrow band barrier filter placed in front of it.

Deep pseudopupil. All measurements were performed on the deep pseudopupil (DPP),† which is a superposition of virtual images of the distal rhabdomere endings (Franceschini and Kirschfeld, 1971). This phenomenon is easily observed when focusing a (low power) microscope deep into the eye, down to the level of its centre of curvature where the magnified images of rhabdomere tips from neighbouring ommatidia are seen as superimposed. The number of ommatidia contributing to the superposition image depends on the aperture of the microscope objective. In acting as a "spatial averager" the deep pseudopupil brings about a substantial improvement in signal-to-noise ratio for any spectroscopic measurement (see Franceschini and Kirschfeld, 1971; Franceschini, 1975, 1983; Stavenga *et al.*, 1973). The measuring diaphragm of the micro-spectrophotometer was closed down to just cover the whole DPP image. This situation would not, in general, yield data from a homogeneous set of receptors, as the photopigments of the central rhabdomeres R7–8 usually differ from that of the peripheral rhabdomeres R1–6.

It is reasonable to assume, however, that any contribution to the optical signals from R7–8 is minor (e.g. Franceschini *et al.*, 1981a). In the male retina there also exists a region where the receptors R7 have photopigment properties identical to those of R1–6 (Franceschini *et al.*,

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†Abbreviations: DPP, deep pseudopupil; M, metarhodopsin; R, rhodopsin.

1981b), and therefore measurements were preferentially executed in that specialized (frontal-dorsal) area (Figs. 3, 6 and 7).

Determination of emission and excitation spectra. Spectral analysis of the fluorescence emission was done under continuous blue or UV excitation by scanning the emitted light with the motorized interference wedge. Any given spectral scan from 400 to 730 nm took 20 s. The resolution of the emission spectra was limited to the bandwidth of the interference wedge, which was about 15 nm at any wavelength. The spectral emission data were corrected by dividing them with spectral data measured from a halogen-tungsten lamp run at 3200 K and standardized using the tabulated emission values of the black body radiator at that temperature. The emission spectrum of M' was determined in two ways: (1) by measuring the emission spectrum of the deep pseudopupil in the blue adapted state and red adapted state, respectively, and then subtracting the second from the first spectrum, (2) by measuring spectra before and after a prolonged UV bleach. The excitation spectrum of M' was determined by (i) measuring the emission from the deep pseudopupil above 665 nm (Kodak Wratten 70) induced by a monochromatic light of a given intensity, (ii) measuring the quantum flux corresponding to that intensity with an

EG & G-radiometer, and (iii) calculating the quantum flux necessary to give a criterion emission. Emission above 665 nm from non-visual pigments was negligible when measuring exclusively from the deep pseudopupil. Our excitation spectrum is plotted as the inverse of this quantum flux at each of the discrete wavelengths chosen.

Photochemistry of housefly visual pigment. The visual pigment of receptors R1–6 can be considered, over a broad range of intensities and exposure times, as a simple photochromic pigment having two photo-interconvertible states. Housefly rhodopsin R490 converts into the metarhodopsin M570 state due to light absorption by R and vice versa. These conversions are most easily studied in the orange where the difference in absorption between R and M is extreme. In the experiment of Fig. 1, 572 nm was taken as the monitoring wavelength so that a decrease in transmission means an increase in metarhodopsin concentration. The changes which are induced by blue (477 nm) and red (613 nm) light result, in the photosteady state, in a high and low metarhodopsin content respectively. For more extensive and formal treatments of various aspects of the photochemistry of invertebrate visual pigments see Stavenga (1976), Hochstein *et al.* (1978), Hamdorf (1979), Cronin and Goldsmith (1982a,b), Hillman *et al.* (1983) and Stavenga and Schwemer (1984).

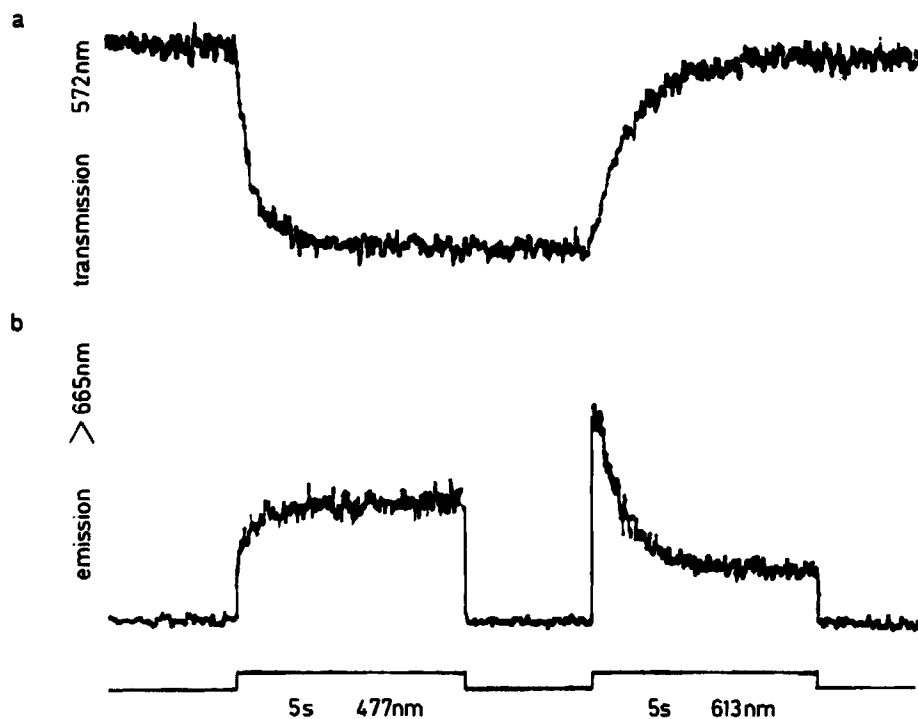


Figure 1. Transmission and fluorescence measurements performed on the eye of the housefly *Musca domestica*, mutant *white*. Antidromic transmission was measured at 572 nm, where absorption by rhodopsin is low and by metarhodopsin is high. Creation of metarhodopsin by orthodromic blue (477 nm) light results in a decrease in transmission (a, left). The orthodromic beam is sufficiently intense to establish a photosteady-state within a few seconds. The high metarhodopsin fraction is maintained after termination of the 5 s lasting orthodromic illumination. The metarhodopsin fraction is lowered by red (613 nm) orthodromic light (a, right). The transmission changes were only about 20% because a major part of the transmission signal is due to stray light. Emission changes (measured above 665 nm) occurring during photoconversion are shown in b. Increase in emission accompanies metarhodopsin creation and a decrease in emission occurs when metarhodopsin is photoreconverted back to rhodopsin. (Metarhodopsin emission is seen superimposed upon a background emission from non-visual pigments in the eye tissue.) The intensities were 5.7×10^{15} quanta $\text{cm}^{-2} \text{s}^{-1}$ (477 nm) and 5.2×10^{15} quanta $\text{cm}^{-2} \text{s}^{-1}$ (613 nm).

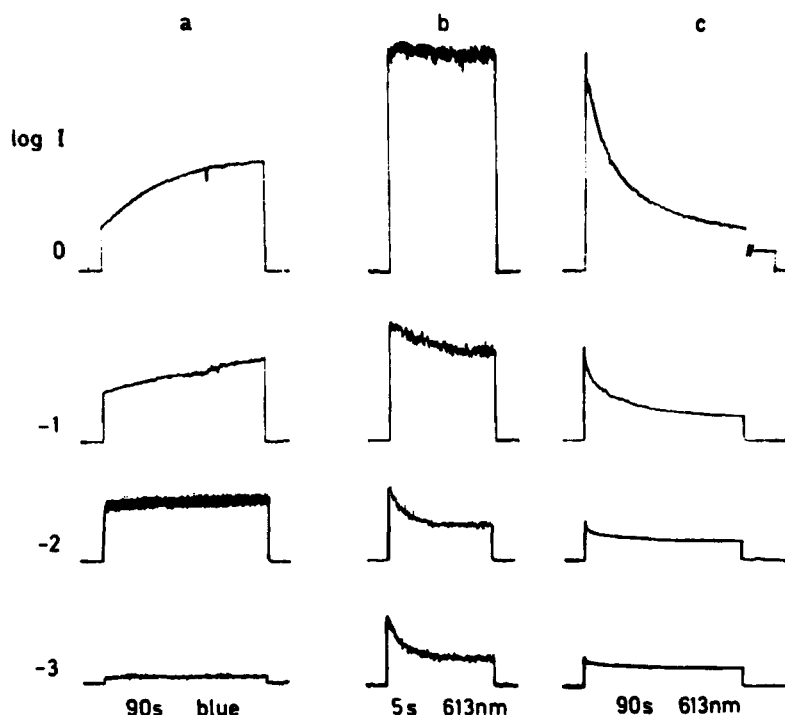


Figure 2. Conversion of visual pigment to metarhodopsin M and M' by blue light (a); illumination time 90 s, intensities 5.8×10^{14} quanta $\text{cm}^{-2} \text{s}^{-1}$ (row -3), 5.3×10^{15} quanta $\text{cm}^{-2} \text{s}^{-1}$ (row -2), 5.1×10^{16} quanta $\text{cm}^{-2} \text{s}^{-1}$ (row -1), 4.3×10^{17} quanta $\text{cm}^{-2} \text{s}^{-1}$ (row 0). The subsequent red (613 nm) flashes, duration 5 s, intensity 5.2×10^{15} quanta $\text{cm}^{-2} \text{s}^{-1}$ (b) show that the blue illuminations result in a high metarhodopsin M content (row -3) and a high metarhodopsin M' content (row 0), respectively. M' is reconverted only after prolonged, intense red light as is demonstrated in (c) where 90 s of 613 nm, intensity 4.7×10^{16} quanta $\text{cm}^{-2} \text{s}^{-1}$ was applied. Full reversion is not achieved in (c) row 0, after 90 s of red illumination, but reversion is complete after another 300 s illumination time; at the end of this time the background emission shown remains again. Always emission > 665 nm was measured. All traces were recorded from the same set of photoreceptors (i.e. the same pseudopupil).

RESULTS

Fluorescence of housefly metarhodopsin M

The photochemical conversion of housefly rhodopsin into metarhodopsin by blue light and the reverse conversion by red light is presented in Fig. 1. The top traces (Fig. 1a) present the antidromic transmission of housefly photoreceptors measured in the deep pseudopupil. Initially all visual molecules exist in the rhodopsin state and illumination with blue light (477 nm) induces conversion of part of the molecules into the metarhodopsin state until a photosteady state is reached. We measured the transmission of the photoreceptors at 572 nm which is near the peak of the metarhodopsin absorption spectrum. The blue induced rise in metarhodopsin hence results in a rise in absorption and thus in a drop in transmission. Illumination of this new state with red light (613 nm) induces reversion of the metarhodopsin molecules into the rhodopsin state, so yielding a drop in absorption (rise in transmission) by the photoreceptors at 572 nm (for very similar

measurements see Fig. 2a of Kirschfeld *et al.*, 1977).

After this experiment we extinguished the antidromic illumination and, in order to measure fluorescence, replaced the 572 nm barrier filter in front of the photomultiplier with a far-red transmitting filter (Wratten 70) and repeated the previous procedure of illumination. Figure 1b demonstrates that a rise in red emission occurs concurrently with the creation of metarhodopsin by blue light, and that a drop in red emission occurs with reversion of metarhodopsin molecules into the rhodopsin state.

The metarhodopsin concentration is negligible at the start of the 477 nm illumination and at the end of the 613 nm illumination. Still the fluorescence value appears to be non-zero. It will become clear below that this fluorescence does not originate from rhodopsin but from non-visual pigments existing in the photoreceptor cells.

Fluorescence of housefly metarhodopsin M'

The visual pigment molecules are converted to the metarhodopsin M' state by intense blue light. The

conversion process is illustrated in Fig. 2. Here the eye of a housefly (mutant *white*) was illuminated during 90 s with broad band blue light (390–490 nm), sequentially at four intensities separated by approximately 1 log unit (Fig. 2a). At the beginning of each of the four illuminations the photopigment was in the fully red adapted state, i.e. all the molecules were in the rhodopsin state according to a control measurement performed as in Fig. 1b. The 90 s of blue light caused conversion of visual pigment, and the resulting pigment composition was then analysed in two steps. First a 5 s red (613 nm; intensity as in Fig. 1) test flash (Fig. 2b) was given, and, subsequently, the red light was made 10 × more intense and applied for another 90 s (Fig. 2c). In all cases the far-red fluorescence (> 665 nm) was measured. The blue illumination with the lowest intensity (Fig. 2a, row –3) induced a substantial conversion of the visual pigment molecules into the metarhodopsin state. This is apparent from the subsequent red test flash which reconverted the metarhodopsin molecules back to the rhodopsin state. We note that during the 5 s of 477 nm light in Fig. 1 2.9×10^{16} quanta cm^{-2} were delivered and a total of 5.2×10^{16} quanta cm^{-2} in the 90 s blue illumination of Fig. 2a, row –3; both are sufficient to establish the photosteady state between rhodopsin R and metarhodopsin M. After the more intense blue illuminations (rows –2, –1) both metarhodopsins M and M' exist, whereas after the brightest blue light (row 0) all molecules appear to be in the M' state.

The photosensitivity for conversion to M' is much smaller than that to M (Fig. 2a,b). The same holds for the opposite process, since M is converted to R within a few seconds in Fig. 2b, whereas the conversion of M' to R requires several minutes even with the ten times more intense red light of Fig. 2c. Furthermore, the recordings of Fig. 2 clearly demonstrate that fluorescence by M' is distinctly more efficient than that by M.

The relatively high efficiency of M' fluorescence, and the ability to create and reconvert this substance by using blue and red illumination respectively, enabled us to determine a relatively uncontaminated M' emission spectrum. Figure 3 presents spectral recordings of the emission induced by broadband blue light (390–490 nm). The initial curve (1) is from a "fresh" animal with as yet virtually no M' present. However, because the blue excitation light was relatively bright to provide an acceptable signal-to-noise ratio, the R–M photosteady state was immediately established; this state remained practically constant during the (20 s) spectral scan. Prolonged exposure to intense blue light resulted in a new pigment composition yielding the blue-adapted spectrum (curve 2). After the subsequent prolonged red illumination, the red-adapted spectrum was recorded (curve 3). Although these spectra are uncorrected, M' clearly appears as a photoreversible substance emitting in the red. Its emission is super-

imposed upon a green photostable emission due to autofluorescence from the cornea and eye tissue, the presence of which is unavoidable, because the (rectangular) measuring diaphragm enclosed an area larger than the pattern of rhabdomere images. Subtracting the emission spectra of the red- from the blue-adapted state (curves 3 and 2 in Fig. 3) eliminates the green autofluorescence component and yields a difference emission spectrum characteristic of substance M'. Correction of this spectrum was done according to the procedures described in the Methods. A clear peak at 660 nm is observed in the corrected emission spectrum of M' (Fig. 4, circles and triangles). No spectral data were obtained above 730 nm due to limitations of the interference wedge.

An excitation spectrum of M' was subsequently determined by monitoring the emission from the deep pseudopupil above 665 nm (see Methods). Figure 5 shows that this spectrum has a distinct maximum at about 570 nm. We have included the emission spectrum of Fig. 4 in order to summarize the fluorescence properties of M'.

Applying ultraviolet (340–380 nm) excitation yields emission spectra with a major blue emission originating from the eye tissue (Fig. 6). Again the existence of the photoconvertible M' photoproduct is clear. The normalized emission difference between the blue and red adapted states indeed coincides with the M' emission spectrum deduced from the blue excitation measurements (Fig. 4). Prolonged intense UV-illumination has dramatic effects, however. The emission irreversibly decreases at all wavelengths and most substantially in the red. M' can be completely bleached away by the intense UV light as well as much of the blue emission.

A measured emission spectrum showing a pronounced contribution from M' is given in Fig. 7a. The destructive effect of UV light is demonstrated by the subsequent substantial decrease of the red emission and a lowered blue emission. The difference in quantum emission between the bright UV adapted and the bleached state is drawn in Fig. 7b, where the dotted curve is obtained by extrapolating the blue peak. The spectrum above this dotted curve is presumed to represent the emission spectrum of M'. Indeed, the normalized spectrum, given by the crosses in Fig. 4 conforms to the photoconversion data.

DISCUSSION

The use of *in vivo* microspectrofluorometry has created a new horizon in the study of the properties of fly photoreceptors. As shown in this report the metarhodopsin of housefly receptors R1–6 displays a distinct red fluorescence, offering a novel opportunity to study the kinetics of visual pigment processes in completely intact, living animals (see also Stavenga, 1983). For example, from the

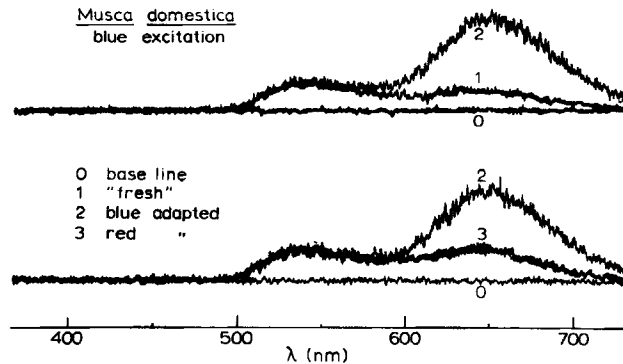


Figure 3. Experimental emission spectra under blue excitation (390–490 nm). Curve 1 is “fresh”, no M' is present, although a substantial amount of M is created already. Prolonged blue light shows a much enhanced emission in the red due to M' formation (curve 2). Subsequent red adaptation reduces the M' concentration, i.e. reduces the emission in the red (curve 3).

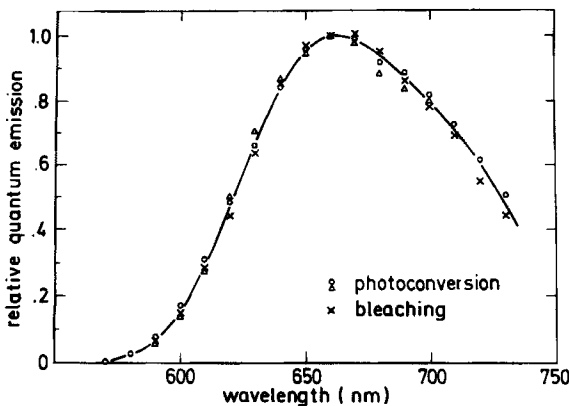


Figure 4. Relative quantum emission of M' . Smoothed data derived from two photoconversion experiments like that of Fig. 3 and from bleaching (Figs. 6 and 7).

dynamics of visual pigment conversion (Figs. 1 and 2) and the applied intensity the relaxation constant can be estimated. (The relaxation constant is the sum of the photosensitivities of rhodopsin and metarhodopsin; Hochstein *et al.*, 1978.) From Fig. 1 we derive that the relaxation constant at 477 nm equals $7 \times 10^{-16} \text{ cm}^2 \text{ quantum}^{-1}$ and at 613 nm $3 \times 10^{-16} \text{ cm}^2 \text{ quantum}^{-1}$. These data are in good agreement with previous estimates (Minke and Kirschfeld, 1979). The numbers must be considered to be rather approximate, however, as a linear proportionality of signal change with metarhodopsin concentration and homogeneity of the retinal tissue has been assumed (see Stark and Johnson, 1980).

The photosensitivities for conversion to and from metarhodopsin M' are clearly much lower than those of M . Why this is so is beyond our present under-

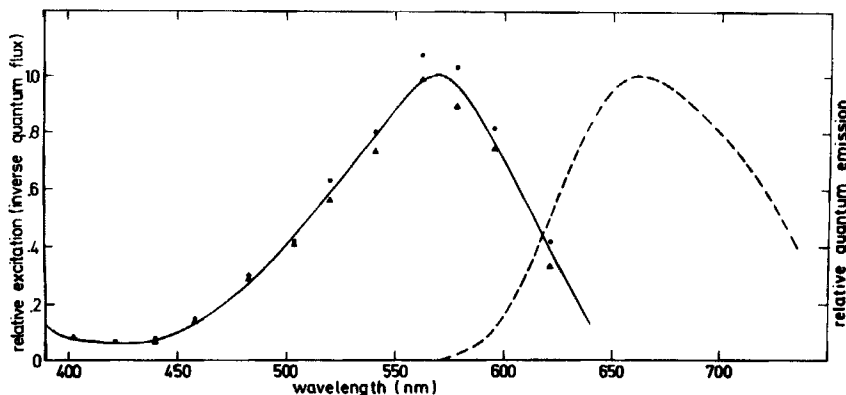


Figure 5. Excitation spectrum of M' determined by calculating the quantum flux to create a criterion emission above 665 nm. The emission spectrum of Fig. 4 is included (dotted curve) thus showing symmetry between excitation and emission spectrum.

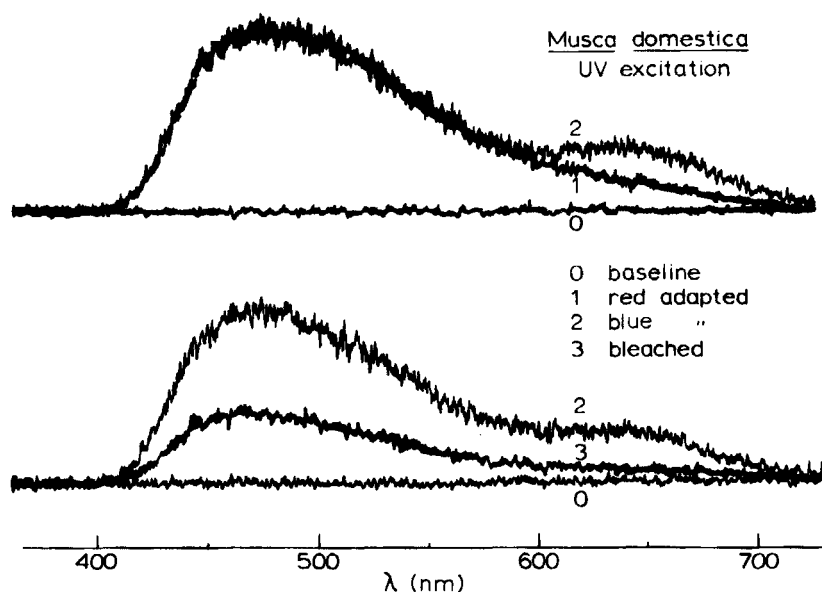


Figure 6. Experimental emission spectra under UV (340–380 nm) excitation. The difference between curve 1 and 2 is due to M' (compare Fig. 3). Prolonged UV light results in irreversible loss of both emission by M' in the red and emission by non-visual pigments in the blue (curve 3). Photodamage during one 20 s scan was negligible.

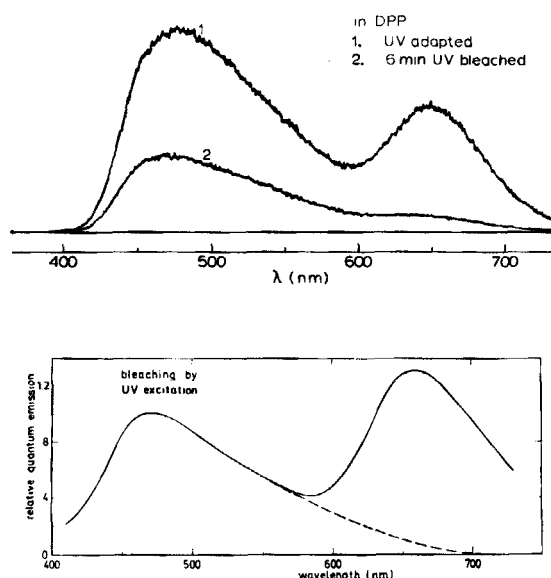


Figure 7. Experimental emission spectra under UV (340–380 nm) excitation before and after bleaching (a, curves 1 and 2). The difference of the curves yields the relative quantum emission (b) due to M' and non-visual pigment which emits maximally at 470 nm.

standing. Also unknown are the photochemical pathways leading to and from M'. Preliminary measurements of excitation and emission spectra of metarhodopsin M demonstrate that the shape of these spectra is very similar to those reported here for

metarhodopsin M' (Fig. 5). Very similar data were recently reported for the metarhodopsin (M) of crayfish rhabdoms by Cronin and Goldsmith (1981, 1982b). Remarkably the emission spectrum of crayfish M is almost coincident with that of fly M' although the excitation spectra are rather different: the maxima are located at 525 nm and 570 nm respectively. As reported above, fly metarhodopsin M emits also in the red range. This is not only found in *Musca* (this study) and *Calliphora* (Stavenga, 1983) but also in *Drosophila* (Stavenga and Stark, unpubl.; Miller *et al.*, 1984). In a previous study on *Drosophila*, metarhodopsin fluorescence remained undetected (Stark *et al.*, 1979), because the emission measurements were not exclusively in the long wavelength range. The contribution to the fluorescence by the non-visual pigments (Figs. 3, 6 and 7) then dominates. Stavenga and Tinbergen (1983) have presented evidence that the shorter-wavelength fluorescence mainly originates from pigments of the mitochondrial respiratory chain. These pigments as well as the visual pigment are deleteriously affected by intense UV light (Stark *et al.*, 1979; Miller *et al.*, 1982; Figs. 6 and 7). In the housefly intense blue lights delivering $\approx 10^{19}$ quanta cm^{-2} convert the visual molecules into the M' state from which reconversion to rhodopsin is possible (Fig. 2; see also Franceschini, 1983, Fig. 8). In the fruitfly, bright blue light of $\approx 10^{20}$ quanta cm^{-2} destroys the visual pigment (Miller *et al.*, 1982; Stark *et al.*, 1983). Considering the extreme brightness of the deleterious illuminations we presume that the

screening pigments in the eyes of wild type flies sufficiently protect the visual sense cells from photodamage.

Acknowledgements—Dr. R. C. Hardie provided substantial support throughout the course of the experiments. Drs. W. S. Bialek, B. Kruizinga, B. Minke and W. S. Stark, as well as two referees, provided valuable comments on various versions of the manuscript. Financial support came from the Netherlands Organization for the Advancement of Pure Research (Z. W. O. to D. G. S.), through the Foundation for Biophysics.

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Note added in proof: Vogt (*Z. Naturforsch.* **38C**, 329–333, 1983; **39C**, 196–197, 1984) and Vogt and Kirschfeld (*Naturwissensch.* **71**, 211–213, 1984) established recently that the chromophore of fly visual pigment is 3-hydroxy-retinal, and not retinal as in the rhodopsins. They propose to call the new class of visual pigments xanthopsins. Accordingly the metarhodopsins of the present paper should be mentioned as metaxanthopsins.